RESEARCH NOTE

Three years of multi-laboratory external quality control for the molecular detection of *Toxoplasma gondii* in amniotic fluid in France

*P. Bastien[1], E. Jumas-Bilak[1,2], E. Varlet-Marie[1] and P. Marty[3], on behalf of the ANOFEL Toxoplasma-PCR Quality Control Group**

[1]Laboratoire de Parasitologie-Mycologie, Laboratoire Associé (Pôle 'Biologie Moléculaire') of the Centre National de Référence de la Toxoplasmose, Centre Hospitalier Universitaire (CHU), Université Montpellier I, [2]Laboratoire de Bactériologie-Virologie (EA3755), Faculté de Pharmacie, Montpellier and [3]Laboratoire de Parasitologie-Mycologie, CHU Nice, France

ABSTRACT

Between 2002 and 2004, panels of amniotic fluid containing varying concentrations of *Toxoplasma gondii* were sent to up to 23 laboratories in France for molecular (PCR-based) detection as part of a national quality assurance initiative in the molecular prenatal diagnosis of toxoplasmosis. Participants were free to enrol and no fees were required. The general level of sensitivity was high, and the rate of false-positive reactions was relatively low. Considerable diversity among PCR methods and primers was revealed. This external quality assurance scheme provided the opportunity to improve laboratory practice and performance, and to increase communication among laboratories involved in making this diagnosis.

Keywords Diagnosis, external quality control, molecular diagnostics, PCR, quality assurance, *Toxoplasma gondii*

Corresponding author and reprint requests: P. Bastien, Laboratoire de Parasitologie-Mycologie, Laboratoire Associé (Pôle 'Biologie Moléculaire') of the Centre National de Référence de la Toxoplasmose, Centre Hospitalier Universitaire (CHU) of Montpellier, 163 Rue Auguste Broussonet, 34000 Montpellier, France
E-mail: p-bastien@chu-montpellier.fr
*P. Bastien, M. H. Bessières, S. Cassaing, A. Bonnin, S. Bretagne, B. Cimon, M. L. Dardé, L. Delhaes, T. H. Duong, D. Filisetti, P. Flori, B. Fortier, I. Villena, J. Franck, J. P. Gangneux, P. Marty, M. Miegeville, P. Millet, L. Paris, H. Pelloux, M. P. Brenier-Pinchart, F. Peyron, D. Quinio, P. Roux, P. Thulliez, M. Vergnaud and H. Yera.

Original Submission: 2 February 2006; **Revised Submission:** 3 September 2006; **Accepted:** 15 September 2006

Clin Microbiol Infect 2007; **13**: 430–433
10.1111/j.1469-0691.2006.01642.x

Toxoplasmosis is an endemic protozoan disease of great public health importance because of possible vertical transmission from an infected woman to a foetus during pregnancy. Prenatal diagnosis of congenital toxoplasmosis has improved the prognosis and outcome for infected children considerably wherever it has been implemented. Prenatal diagnosis has been a national policy in France since 1978 [1]. PCR-based molecular diagnostic tests using amniotic fluid (AF) have largely superseded more classical methods, and have eliminated the need for cordocentesis [2]. However, all PCR assays used for this application are currently in-house methods established independently in each laboratory. This is known to lead to considerable variation in diagnostic performance. Accordingly, with a view to improving and standardising prenatal diagnosis at a national level, an external quality assessment (EQA) scheme was established during 2002 in France, under the auspices of the national association of hospital practitioners and teachers in parasitology–mycology (ANOFEL). This EQA was repeated in 2003 and 2004. Participating laboratories were free to enrol, anonymity of results was guaranteed, and no fees were required for participation. The number of participants in the scheme increased from 21 in 2002 to 23 in 2004. The test material included negative and positive samples of AF containing different *Toxoplasma* concentrations. Only low concentrations were used, (i) because most diagnostic methods for pathogens are known to be particularly fallible with low concentrations of pathogens in the sample [3,4], and (ii) because it has been established that a notable proportion of infected AFs contain low *Toxoplasma* loads [5].

The test material was based on naturally infected samples. Signed consent was obtained from all women from whom the samples were obtained. Known PCR-positive and PCR-negative AFs were pooled separately, and the pools were then tested for the presence of bacterial

contamination and by *Toxoplasma* PCR (four and eight reactions per pool, respectively). The reference PCR assay used to control negative samples allowed the detection of 0.5–1 parasites/mL [6]. The negative pool was then used for making negative control samples, and also for diluting the positive pool to produce samples with different *Toxoplasma* concentrations. Compared with the more classical method of seeding negative AFs with *Toxoplasma* tachyzoites drawn from mouse ascitis, the method was simple and used naturally infecting strains, but had the disadvantage of using thawed samples and of, theoretically, being less precise in the quantification of parasites. In the present study, parasite concentrations in the positive pool were estimated by quantitative PCR in two or three different laboratories before dilution and distribution.

Depending on the year, panels included two to five samples, totalling 245 samples during the 3-year period. Each participant received identical 2-mL samples drawn at the same time from the same pools. All participants responded, and results were analysed anonymously. All participating laboratories used in-house PCR assays. The DNA extraction methods and PCR primers used varied considerably among laboratories (Table 1). The overall results are shown in Table 2. Sensitivity problems were observed only for very low parasite concentrations (<10/mL); below this threshold, two to four of the 21–23 laboratories reported false-negative results, representing 6.7% of 134 samples, which is a low rate of false-negative results. Thus, the overall level of sensitivity among the participating laboratories appeared to be high. In comparison, a study in 1998 reported that 40% of samples with a concentration of 10 tachyzoites/mL generated false-negative results [3]. More recently, an international study involving 33 laboratories (of which none appear to be included in the French network) reported 19.5% false-negative results (vs. 6.7% in the present study) [4]. The false-negative results in the present study did not appear to be related to a particular PCR method (e.g., real-time, DNA target or primer pair), but can be attributed to technical proficiency, PCR optimisation and laboratory practices. Identical conclusions were reached by Kaiser *et al*. [4].

It should also be noted that no DNA stabiliser was used at any stage. However, the absence of *Toxoplasma* DNA degradation in the samples was checked: (i) by multiple PCR testing before sending the samples; and (ii) after sending the samples, by keeping an entire panel at room temperature for 5 days in the coordinating laboratory before DNA extraction and PCR testing. However, it is possible that an undetectable level of DNA degradation might contribute to the false-negative results observed for samples with very low parasite concentrations (e.g., 2–6/mL), and in laboratories with a slightly less sensitive PCR assay.

Table 1. Overview of the methods and primers used in the French external quality assessment scheme for molecular detection of *Toxoplasma gondii* in 2004

DNA extraction method (*n*)	PCR-based method (*n*)	DNA target[a] (*n*)	Primers used[a] (*n*)
TNN method[b], 4	Conventional, 12	B1 gene, 17	B22–B23 [12], 6
Commercial kit, 19	Real-time, 13		T1–T4 [13], 2
Qiagen[c], 13	LightCycler (Roche), 6		F–R [14], 2
Roche[d], 4	Applied Biosytems, 4		[5], 2
Epicentre[e], 1	i-Cycler (BioRad), 1		B5–B6 [15], 1
Not known, 1	Mx4000 (Stratagene), 1		JW58–59 [16], 1
	RotorGene (Corbett), 1		Unpublished, 2
			Not known, 1
		REP529[f], 12	[9], 5
			TOX4-5 [17], 2
			[18], 2
			Unpublished, 3
		rDNA[g], 2	[19], 2

[a]The number of DNA targets and primer pairs may differ from that of participants, as several laboratories used two different PCR methods.
[b]TNN (Tween–Nonidet–NaOH), a simple DNA isolation method described by Hohlfeld *et al*. [20].
[c]Qiagen QIAmp DNA mini-kit.
[d]Roche HighPure PCR Template Kit.
[e]Epicentre MasterPure DNA.
[f]Repetitive non-coding sequence identified by Homan *et al*. [17].
[g]Ribosomal DNA gene.

Table 2. Overall results for the molecular detection of *Toxoplasma gondii* in the French external quality assessment in 2002–2004

Date	Samples[a]	*n*	False-positive[b]	False-negative[c]
2002	–	21	0	
	+ (6–10/mL)	21		4
2003	–	22	1	
	–	22	1	
	+ (2–6/mL)	22		3
	+ (10–20/mL)	22		0
2004	–	23	1	
	–	23	0	
	+ (4–8/mL)	23		1
	+ (8–16/mL)	23		1
	+ (30–60/mL)	23		0
Total	–	111	3	
	+	134		9

[a] –, negative sample; +, positive sample (estimated parasite concentration in tachyzoites/mL; see text).
[b]Number of negative samples found to be positive.
[c]Number of positive samples found to be negative.

With regard to specificity, the cumulative rate of false-positive results (2.7%; three of 111 samples) was also relatively low in comparison with other molecular EQA programmes, e.g., 2–10% [4,7], 11.6% [3], or 35% [8]. However, their presence stresses the need to constantly re-evaluate protocols for the prevention of carryover contamination. The multiplicity of replicates of negative controls may help to reveal more contaminations of this type, which tend to be sporadic in nature. As negative AF samples were drawn from women who had seroconverted during pregnancy, an alternative explanation for false-positive results could involve the presence of extremely low concentrations of parasites in these samples that would go undetected by most participants. Assuming that most *Toxoplasma* PCR assays have a median sensitivity of *c*. 5, or 1–10, parasites/mL [6,9], parasite concentrations giving such results would be <5/44–46 mL, i.e., approximately <1 parasite/10 mL, which is feasible from the pathophysiological viewpoint. However, this seems unlikely, as a 1-year follow-up of the infants of these infected women did not reveal congenital infection after birth.

The overall performance of this national network was better than that reported by three previous international inter-laboratory evaluations [3,4,10]. In particular, the most recent evaluation [4] reported correct results in the entire panel for only 42.1% of datasets, compared with 95.1% in the present study. It is noteworthy that the laboratories which provided incorrect results in the present EQAs were different on each occasion, indicating the likely absence of major systematic faults during the diagnostic process in a particular laboratory. This correlates well with the absence of any association between the methods used and false-negative or false-positive results, as noted previously [4]. This also highlights the need for constant evaluation and vigilance in all diagnostic laboratories using PCR. Finally, it is important to stress that this EQA scheme, as well as the way in which it was conducted, had positive consequences for molecular diagnosis of toxoplasmosis in France. First, it may have improved the general detection level, in view of the decrease over the 3-year period in the rate of false-negative results from 19% (4/21) to 4% (1/23), for parasite concentrations of <10/mL. Second, recommendations made after each EQA led to an increase in 'approved' practices for molecular diagnosis. Third, it has encouraged a new spirit of communication and exchange among the French laboratories involved in making this diagnosis.

In recent years, several inter-laboratory comparative studies have been described for the molecular diagnosis of toxoplasmosis [3,4,10,11]. All of these studies underlined the urgent need for standardisation of PCR protocols and, in this context, the conclusions of Pelloux *et al*. [3] remain remarkably topical. The present national scheme in France is a first and necessary step towards this difficult goal. Standardisation of quantitative PCR methods and results should also be included in view of the growing number of laboratories using such methods.

ACKNOWLEDGEMENTS

We thank all the members of the ANOFEL Toxoplasma Quality Control Group for their participation, and ANOFEL for financial support. We are particularly indebted to P. Thulliez and H. Pelloux, who supplied some of the positive and negative AFs used for constituting the artificial samples used in this study, and to I. Villena and D. Filisetti, who helped in parasite quantification. We thank I. Villena for fruitful discussions and are indebted to D. Chabasse and J. M. Pinon for their support of the EQA initiative. We also acknowledge the assistance of E. Chabbert and the technical help of G. Bresson, S. Douzou, L. Sanichanh and F. Michel.

REFERENCES

1. Thulliez P. Screening programme for congenital toxoplasmosis in France. *Scand J Infect Dis* 1992; **84** (suppl): 43–45.
2. Bastien P. Molecular diagnosis of toxoplasmosis. *Trans R Soc Trop Med Hyg* 2002; **96** (suppl 1): S205–S215.
3. Pelloux H, Guy E, Angelici MC *et al*. A second European collaborative study on polymerase chain reaction for *Toxoplasma gondii*, involving 15 teams. *FEMS Microbiol Lett* 1998; **165**: 231–237.
4. Kaiser K, Van Loon AM, Pelloux H *et al*. Multicenter proficiency study for detection of *Toxoplasma gondii* in amniotic fluid by nucleic acid amplification methods. *Clin Chim Acta* 2007; **375**: 99–103.
5. Costa JM, Ernault P, Gautier E, Bretagne S. Prenatal diagnosis of congenital toxoplasmosis by duplex real-time PCR using fluorescence resonance energy transfer hybridization probes. *Prenat Diagn* 2001; **21**: 85–88.
6. Chabbert E, Lachaud L, Crobu L, Bastien P. Comparison of two widely used PCR primer systems for detection of *Toxoplasma* in amniotic fluid, blood and tissues. *J Clin Microbiol* 2004; **42**: 1719–1722.

7. Walton C, Hawkey PM, James VL. Examination of specimens for mycobacteria in clinical laboratories in 21 countries: a 10-year review of the UK National Quality Assessment Scheme for mycobacteria culture. *Clin Microbiol Infect* 2005; **11**: 1016–1021.
8. Valentine-Thon E, van Loon AM, Schirm J, Reid J, Klapper PE, Cleator GM. European proficiency testing program for molecular detection and quantitation of hepatitis B virus DNA. *J Clin Microbiol* 2001; **39**: 4407–4412.
9. Reischl U, Bretagne S, Kruger D, Ernault P, Costa JM. Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infect Dis* 2003; **2**: 3–7.
10. Guy EC, Pelloux H, Lappalainen M *et al.* Interlaboratory comparison of polymerase chain reaction for the detection of *Toxoplasma gondii* DNA added to samples of amniotic fluid. *Eur J Clin Microbiol* 1996; **15**: 836–839.
11. Costa JM, Munoz C, Krüger D *et al.* Quality control for the diagnosis of *Toxoplasma gondii* reactivation in SCT patients using PCR assays. *Bone Marrow Transplant* 2001; **28**: 527–528.
12. Bretagne S, Costa JM, Vidaud M, Tran J, Nhieu V, Fleury-Feith J. Detection of *Toxoplasma gondii* by competitive DNA amplification of bronchoalveolar lavage samples. *J Infect Dis* 1993; **168**: 1585–1588.
13. Burg JL, Grover CM, Pouletty P, Boothroyd JC. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol* 1989; **27**: 1787–1792.
14. Lin MH, Chen TC, Kuo TT, Tseng CC, Tseng CP. Real-time PCR for quantitative detection of *Toxoplasma gondii*. *J Clin Microbiol* 2000; **38**: 4121–4125.
15. Dupouy-Camet J, de Souza SL, Maslo C *et al.* Detection of *Toxoplasma gondii* in venous blood from AIDS patients by polymerase chain reaction. *J Clin Microbiol* 1993; **31**: 1866–1869.
16. Pelloux H, Weiss J, Simon J *et al.* A new set of primers for the detection of *Toxoplasma gondii* in amniotic fluid using polymerase chain reaction. *FEMS Microbiol Lett* 1996; **138**: 11–15.
17. Homan WL, Vercammen M, De Braekeleer J, Verschueren H. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *Int J Parasitol* 2000; **30**: 69–75.
18. Cassaing S, Bessières MH, Berry A, Berrebi A, Fabre R, Magnaval JF. Comparison between two amplifications for molecular diagnosis of toxoplasmosis by real-time PCR. *J Clin Microbiol* 2006; **44**: 720–724.
19. Cazenave J, Cheyrou A, Blouin P, Johnson AM, Begueret J. Use of polymerase chain reaction to detect *Toxoplasma*. *J Clin Pathol* 1991; **44**: 1037.
20. Hohlfeld P, Daffos F, Costa JM, Thulliez P, Forestier F, Vidaud M. Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. *N Engl J Med* 1994; **331**: 695–699.

RESEARCH NOTE

Release of granzymes and chemokines in Thai patients with leptospirosis

M. De Fost[1], W. Chierakul[2], R. Limpaiboon[3], A. Dondorp[2,4], N. J. White[2,4] and T. Van Der Poll[1]

[1]Centre for Infection and Immunity Amsterdam, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands, [2]Faculty of Tropical Medicine, Mahidol University, Bangkok, [3]Department of Medicine, Udon Thani General Hospital, Udon Thani, Thailand and [4]Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, University of Oxford, Oxford, UK

ABSTRACT

The plasma concentrations of granzymes are considered to reflect the involvement of cytotoxic T-cells and natural killer cells in various disease states. Interferon (IFN)-γ-inducible protein-10 (IP-10) and monokine induced by IFN-γ (Mig) are members of the non-ELR CXC chemokine family that act on T-cells and natural killer cells. This study revealed that the plasma concentrations of granzyme B (but not granzyme A), IP-10 and Mig were higher in 44 Thai patients with definite or possible leptospirosis than in healthy blood donors. These data suggest that activation of cell-mediated immunity is part of the early host response to leptospirosis.

Keywords Cell-mediated immunity, chemokines, granzymes, host reponse, leptospirosis

Original Submission: 1 June 2006; **Revised Submission:** 24 August 2006; **Accepted:** 21 September 2006

Clin Microbiol Infect 2007; **13**: 433–436
10.1111/j.1469-0691.2006.01640.x

Leptospirosis is caused by pathogenic spirochaetes of the genus *Leptospira*, and is probably the world's most widespread zoonosis [1]. Cytotoxic T-cells

Corresponding author and reprint requests: T. van der Poll, Academic Medical Centre, University of Amsterdam, Meibergdreef 9, Room G2-130, 1105AZ Amsterdam, The Netherlands
E-mail: t.vanderpoll@amc.uva.nl